ALKALINE PHOSPHATASE AND VITAMIN C DEFICIENCY IN REGENERATION OF SKULL BONES

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It has been shown that the fibres produced in healing wounds in the femora of guinea-pigs (Bourne, 1943*a*) and in skin wounds (Fell & Danielli, 1943) appear to contain alkaline phosphatase (technique of demonstration described by Gomori, 1941). It has also been shown (Bourne, 1943*b*) that in scurvy in guinea-pigs there is a decrease in the area containing alkaline phosphatase at the costo-chondral junctions.

In view of these facts it was decided to investigate histologically in greater detail the distribution of phosphatase in the various cells concerned in the healing process in holes bored in the skulls of normal and partly scorbutic guinea-pigs. The skull was chosen because the bone is fairly thin and it is possible therefore to cut undecalcified sections (and thus not to interfere with the phosphatase reaction or vital staining) if the tissue is embedded in sufficiently hard celloidin. Macrophages were differentiated from osteogenetic and other cells by intravital staining with trypan blue.

METHOD

Sixteen male guinea-pigs, weighing between 200 and 300 g. each, were used in this investigation. They were given the following diets:

A. Eight were placed on a normal diet. (Rat cake, greens, *ad lib.*, 2 drops of vitamin A and D concentrate twice a week. For the composition of the rat cake see Bourne, 1942*b*.)

B. Four were placed on a scorbutic diet but received in addition a daily supplement each by mouth of 10 mg. of synthetic vitamin C freshly dissolved in distilled water for 1 week before operation.

C. Four were placed on a scorbutic diet (as described by Bourne, 1942*a*) for 1 week before operation.

These three groups provided an opportunity of comparing the healing processes in scorbutic animals and animals on a normal diet, and also provided information as to whether pure synthetic vitamin C was as effective in facilitating the healing process as a normal diet of greens.

In every animal two holes 2 mm. in diameter were bored in the parietal bones of the skull and in two animals of groups B and C two holes 2 mm. in diameter were bored in the femora also.

In group A the animals were killed at the following times: two at 24 hr. after operation; two at 3 days; two at 1 week; and two at 2 weeks after operation.

This gave an opportunity of observing the various stages of healing in an injury to the skull.

Groups B and C were both killed on the 4th day after operation. The animals of these groups were, in addition, given a daily intraperitoneal injection of a 1 % solution of trypan blue at the rate of 1 c.c. per 100 g. of body weight.

All the tissues (pieces of parietal bone and of femur) were fixed immediately after death in ice-cold 80 % alcohol. Most of the pieces of skull were embedded in celloidin and undecalcified transverse sections 50 μ thick were cut from them. Some pieces of skull were decalcified in a vitamin C solution in the hope that paraffin sections could be obtained which would still contain active phosphatase, but without success. (Vitamin C if used in the dark, is a relatively good decalcifying agent. A 5 % solution has half the activity of a 5 % solution of trichloracetic acid.) After the femora of the animals had been fixed for 24 hr. the repair tissue in the holes was scooped out and sectioned in the ordinary way. All sections were treated by Gomori's (1941) alkaline phosphatase technique.

RESULTS

A. *The healing process in the skulls of animals on a normal diet*

24 hr. Two main types of phosphatase positive cell were present in the blood clot filling the hole in the skull. The first type gave an intensely positive phosphatase reaction in the nucleus (Pl. 1, fig. 1), but the cytoplasm of some of the cells contained numerous dark granules, presumably representing sites of phosphatase activity, which made it impossible to distinguish the nucleus. These cells appeared to be polymorph leucocytes. The second type of cell gave a strong reaction in both nucleus and cytoplasm, and possessed elongated processes which also appeared to contain the enzyme (Pl. 1, fig. 2). In the central part of the hole other phosphatase positive cells resembling in general the second type appeared to join up to form capillaries.

Large numbers of the first type of cell were also present in both periosteum and endosteum; more were present in the former than in the latter. Not all of these cells in the periosteum stained in this way, but the number which gave a positive phosphatase reaction increased near the margins of the hole. About 4 mm. from the hole some eight out of every twenty polymorph-type cells in the periosteum were phosphatase positive (Pl. 1, fig. 3). At 3 mm. from the hole about fifteen out of twenty cells were positive. This was the same up to 0·5 mm. from the hole, but from there to the margin of the hole every cell was phosphatase positive. Some of the cells in the periosteum in which the whole nucleus had not blackened showed an intensely positive nucleolus. This increase in the number of phosphatase positive cells in the periosteum suggests that they migrated along it and that as they came closer to the area of injury they were under the influence of some chemical substance liberated from the injured tissue which caused them either to synthesize alkaline phosphatase or to absorb it.

3 *days*. There were fewer of the polymorph leucocyte type of cells in the injured area or in the periosteum and endosteum. Numerous osteogenetic fibres were present in the hole and in the cellular layers of the periosteum near the hole. Many of these fibres gave a positive phosphatase reaction (Pl. 1, fig. 5). Most of the nuclei of the periosteal cells (cambial layer) gave a slight positive phosphatase reaction. More phosphatase positive capillaries were present.

1 *week*. Two massive concentrations of phosphatase were present, one on either side of the repair tissue, about half-way between the outer and inner surfaces of the hole, and there were histological signs in these regions that formation of trabeculae was commencing (Pl. 1, fig. 6). There were practically no phosphatase positive cells in the periosteum at this stage.

2 *weeks*. The numerous fibres in the repair tissue gave a positive phosphatase reaction and in certain areas—particularly where the concentration of phosphatase was heaviest, bony trabeculae could be seen forming. None of the positive polymorph-type cells, seen so plentifully in the 24 hr. preparation, could be seen in the repair tissue or in the periosteum.

B. *Animals on a scorbutic diet plus synthetic vitamin C*

The animals of this, and the succeeding, group were given trypan blue as described above. All four animals in this group were killed 4 days after operation. Numerous osteogenetic fibres were present in the repair tissue in most specimens. A large number of polymorph-type cells were present but only a small proportion of them had phosphatase positive nuclei. Numerous macrophages (detectable by their reaction to trypan blue, see Cappell, 1929) were present in the repair tissue, and in the endosteum and periosteum. Only a few were present in the cellular layer of the periosteum (Pl. 1, fig. 4; Pl. 2, figs. 7, 8). In one preparation a periosteal blood vessel was cut through near the margin of the hole and macrophages could be seen in the vessel. Macrophages could be seen outside the vessel also giving the impression that they were migrating out into the fibrous layer of the periosteum and then along this, guided by the fibres of that layer, towards the hole. Accumulations of macrophages could also be seen in some endosteal blood vessels and in some blood vessels in the bone itself (Pl. 2, fig. 9). So many were present in some of the vessels that near the injury the whole of the contents of the vessel appeared to stain a bright blue. It is noteworthy that the macrophages gave no phosphatase reaction.

C. *Animals on a scorbutic diet*

All four animals in this group were killed 4 days after operation. In all specimens relatively few cells were present in the repair tissue filling the hole. There were some rounded cells containing coarse granules of trypan blue which gave no phosphatase reaction (these were macrophages). There were some cells with elongated processes some of which gave a slight phosphatase

reaction and some which gave no reaction at all. A few polymorph cells were present which gave a positive reaction. All preparations showed a few fine fibres and capillaries which were also positive. Near the edges of the hole where there was some necrosis and crumbling of bone, macrophages could be seen which contained masses of material that stained black with cobalt sulphide without prior incubation with enzyme substrate. This material was probably necrotic bone. It is of interest that the osteocytes in the uninjured bone do not take up trypan blue to any extent. Sections of material from the hole bored in the femur also showed a reduction in the number of cells present in the injured area in scorbutic animals. In bone marrow of both normal and scorbutic animals bone marrow cells gave a positive phosphatase reaction, and macrophages (stained with trypan blue but giving no phosphatase reaction), could be seen among them (Pl. 2, fig. 10).

DISCUSSION

This work shows that within 24 hr. of injury to a skull bone there is an accumulation of cells, the nuclei of which give a positive phosphatase reaction, in the injured area and in the periosteum and endosteum near the region of injury. The cells are similar to those found by Fell & Danielli (1943) in injuries to the skin and called by them polymorphs. The polymorphs present in the injured area probably come from blood vessels in the periosteum and it is only when they approach the site of injury that they give a strong positive phosphatase reaction. The number of these cells migrating to the injured area in the skull bone is very greatly reduced in scorbutic animals, but the ability of the cells which do get to the injured area to give a positive phosphatase reaction does not appear to be affected. Vitamin C deficiency appears therefore to reduce the migratory powers of polymorphs, but does not completely inhibit the production of some substance from the injured area which apparently stimulates the production or absorption of phosphatase by the polymorph.

Osteogenetic cells and fibres give a positive phosphatase reaction when they first appear in an injured region. As the formation of trabeculae commences there is further and more intense accumulation of phosphatase in the cells and fibres at the site of formation of trabeculae.

There is thus a double cycle of phosphatase production by osteogenetic cells in the process of bone formation. The first when the osteogenetic fibres are produced, and the second when the fibres are collected together as trabeculae. A scorbutic diet not only reduces the number of polymorphs present in the injured area but also has an inhibiting effect on the production of fibres by the osteoblasts and reduces the number of capillary vessels formed.

It is of interest that Willmer (1942) found that normal fibroblasts, endothelial cells and macrophages in tissue culture could not be distinguished from one another by the phosphatase reaction, which is weak in all of them unless they are undergoing mitosis. In the present work, cells which appear to be forming capillaries (endothelial cells) and those forming fibres (osteogenetic

cells, which are a special type of fibroblast) gave similar phosphatase reactions. On the other hand, macrophages gave an almost completely negative reaction, although in some of them just the slightest blackening of the nuclear membrane could be seen. One must remember, however, that the conditions under which Willmer examined his cells (in tissue culture) were very different from the conditions under which the present author studied the same cells and the differences in staining reactions are almost certainly due to this fact.

The flood of macrophages which pours into the injured area confirms more indirect observations of the same phenomenon made by Macklin (1920). There is no evidence that these macrophages are formed by transformation of periosteal cells or modification of fibroblasts or other cells in the clot. Macrophages in the periosteum near the injury were concentrated in the fibrous layer; there were only a few in the cellular layer. They could be seen in periosteal blood vessels and in blood vessels in the bone. In the latter there was a very large concentration of them near the injury. While this is scarcely the place to enter into a discussion of the origin of macrophages, it might perhaps be said that the impression given by the preparations is that most, if not all, of the macrophages come to the injured area via the blood vessels. This agrees with the results of Eberth & Florey (1939).

There are fewer macrophages in the injured area in the vitamin C-deficient animals. This is in accord with previous observations that phagocytosis is inhibited in scurvy, e.g. Hunt (1941) found a delayed removal of catgut ligatures and of damaged muscle in vitamin C deficiency. Matzner (1938) has shown that macrophages in the lung in infections accumulate large stores of vitamin C within themselves so that the vitamin is presumably essential for their activity.

SUMMARY

This work has shown that within 24 hr. of injury to a bone, cells of which the nucleus gives a strong positive phosphatase reaction, and which are probably polymorphs, accumulate in the injured area and in the periosteum and endosteum near it.

Proximity to the injured area appears to accentuate the phosphatase reaction in these cells.

By 3 or 4 days after injury there are very few of these cells left of the injured area and they have almost completely disappeared after 1 week.

Numerous osteogenetic and endothelial cells both of which give a positive phosphatase reaction are present in the injured area within 3 days of the injury.

Macrophages, which give no phosphatase reaction, are present in large numbers in the injured area at 4 days after injury.

The number of all varieties of cells present in the injured area after 4 days is greatly reduced by a scorbutic diet.

The healing process in the skull is very much slower than in the femur. A small hole bored in the femur of a guinea-pig on a normal diet is completely filled with bony trabeculae at the end of 1 week. In the skull only a few trabecular strands are present at the end of 2 weeks.

I am indebted to Roche Products for the supply of synthetic vitamin C used in these experiments.

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EXPLANATION OF PLATES

All photographs (except Pl. 2, fig. 10) are taken from undecalcified sections approximately 50 μ thick. It was found impossible to cut satisfactory undecalcified sections of the guinea-pig's skull at less than this thickness.

PLATE 1

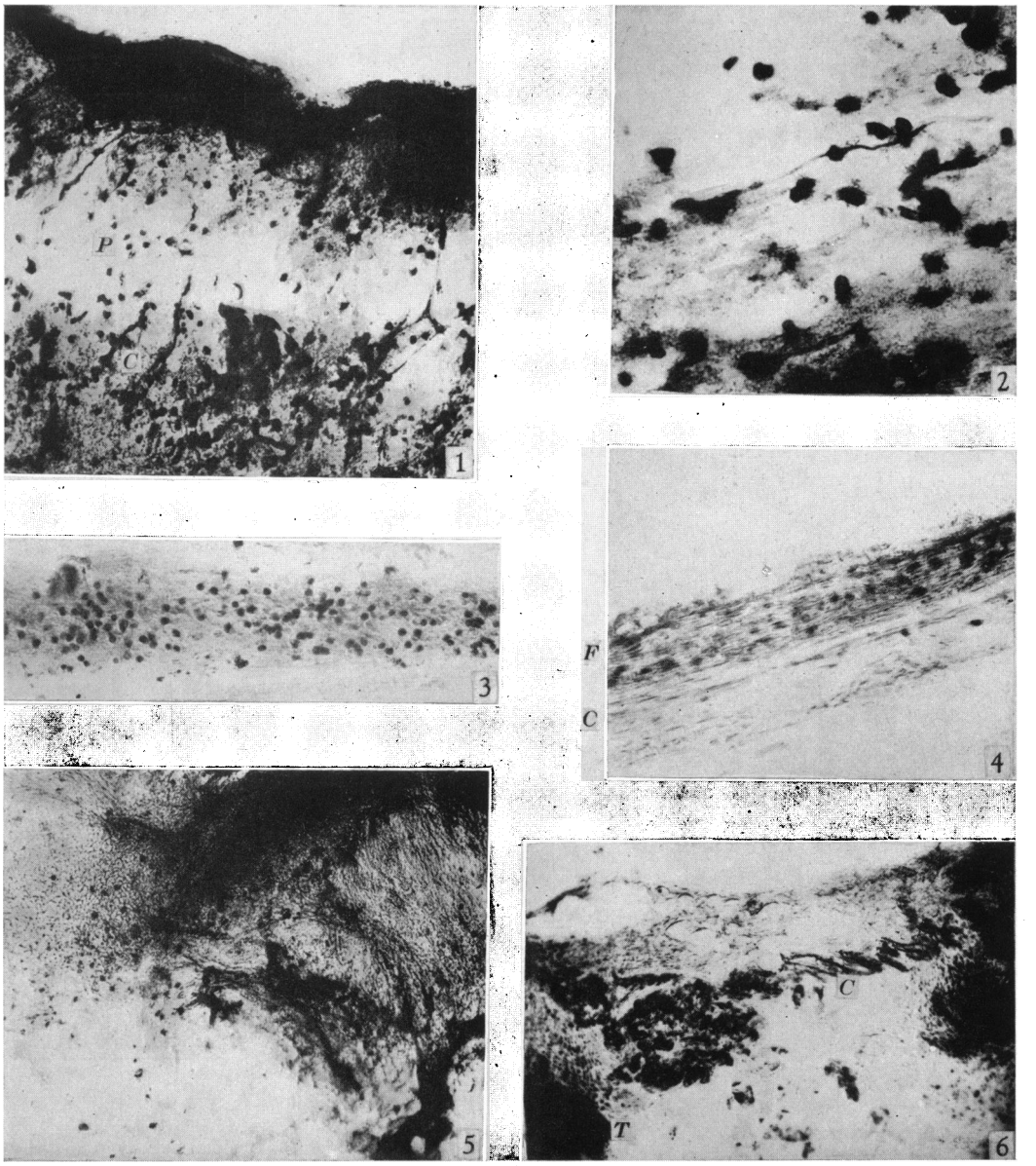
Fig. 1. Repair tissue in hole in skull 24 hr. after operation: outer surface of bone uppermost. Numerous phosphatase positive cells (*P*) are present. A phosphatase positive capillary (*C*) can be seen in process of formation. The black mass at the top of the photograph is due to a fold in the section. (Phosphatase preparation. No counterstain.) × 65.

Fig. 2. Repair tissue from hole in skull 24 hr. after operation. Cells with nucleus and cytoplasm stained black can be seen. A blackened process (? osteogenetic fibre) can be seen extending some 40–50 μ from one cell. This suggests the production of a phosphatase laden fibre from a cell containing large amounts of the enzyme. Other cells with phosphatase mainly in the nucleus can also be seen in the photograph. × 650.

Fig. 3. Cellular layer of periosteum of skull about 4 mm. from hole. 24 hr. after operation. Large numbers of phosphatase positive cells (polymorphs) are present. × 120.

Fig. 4. Periosteum near hole in skull 4 days after operation, showing accumulation of macrophages in fibrous layer (*F*), but only occasional macrophages in cellular layer (*C*). (Intravital trypan blue.) × 120.

Fig. 5. Repair tissue in hole in skull 3 days after operation. Central black mass is formed of phosphatase positive fibres which can be seen more clearly on the right of the photograph. Only a few phosphatase positive cells are present. × 120.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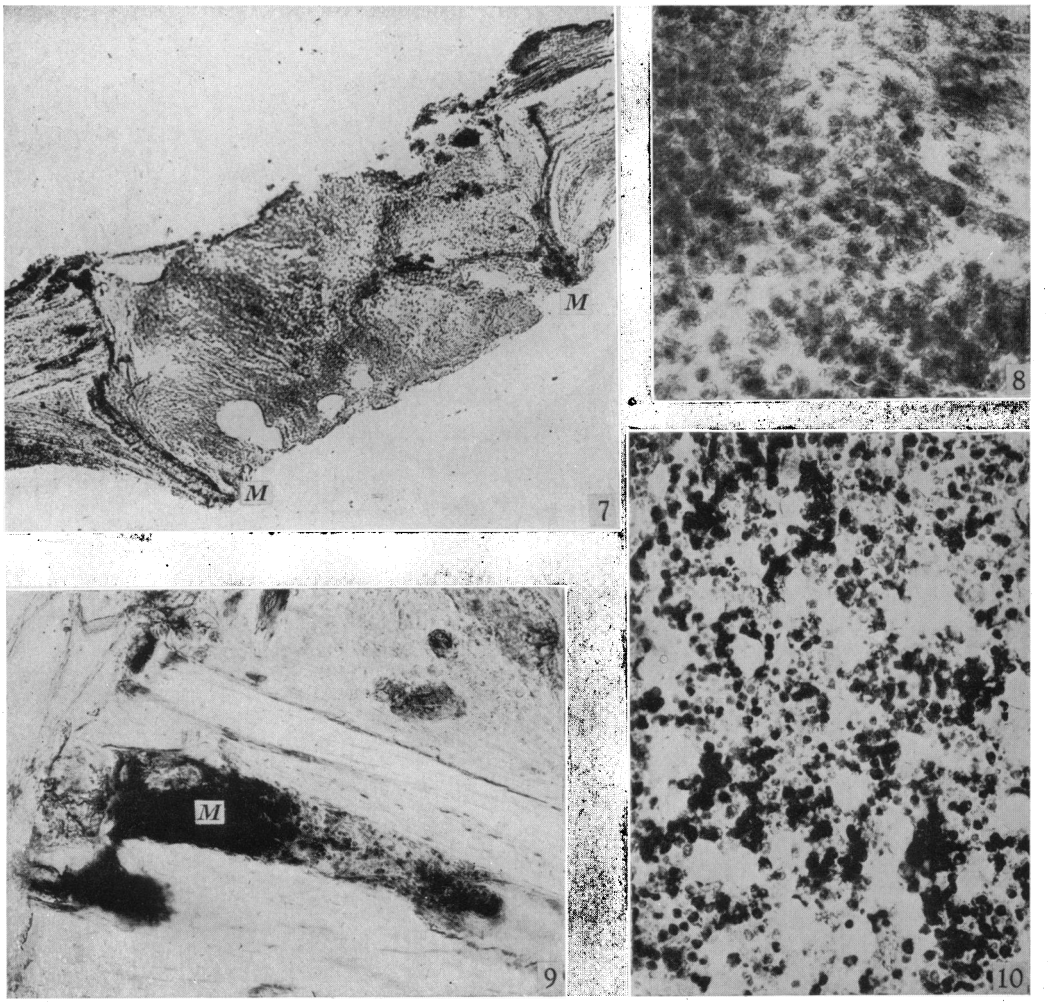

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Fig. 6. Repair tissue in hole in skull 1 week after operation: outer surface of bone uppermost. Black material extending across near the top of the photograph is made up of necrosing chips of bone (*C*). The lateral black masses indicate the formation of bony trabeculae (*T*). That is, the cells in these areas are commencing a second cycle of phosphatase production. Isolated phosphatase positive cells can be seen at the margins of these black masses. ×65.

PLATE 2

Fig. 7. Hole in skull 4 days after operation: outer surface of bone uppermost. Dark areas in repair tissue and at margins of hole (*M*) represent aggregations of macrophages. The only stain used is intravital trypan blue. ×65.

Fig. 8. Repair tissue from hole in skull 4 days after operation, showing masses of macrophages. The granular character of the cells can be seen. (Intravital trypan blue.) ×250.

Fig. 9. Blood vessel near hole in skull 4 days after operation. On the left macrophages are so numerous in the vessel as to appear as a black mass (*M*), but are not so numerous on the right and so individual cells can be seen. (Intravital trypan blue.) ×50.

Fig. 10. Repair tissue from hole in femur. Phosphatase positive cells appear black. Macrophages, which give a negative phosphatase reaction, have trypan blue granules in the cytoplasm and appear grey in the figure. (Intravital trypan blue and phosphatase technique.) ×100.